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14. ABSTRACT
The association between SNCG expression and the progression of steroid dependent cancers of breast and ovary promoted us to investigate the role of SNCG in regulation of ERα. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells. A notable finding relevant to this study is that SNCG, acting as a chaperone for ER, strongly stimulated the ligand-dependent transcriptional activity of ERα, ligand-dependent cell growth, and ligand-dependent mammary tumorigenesis. Augmentation of SNCG expression stimulated the transcriptional activity of ERα and ligand-dependent growth, whereas compromising endogenous SNCG expression suppressed ERα signaling and ligand-dependent growth.

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INTRODUCTION

SNCG was first identified and cloned in PI's lab as a breast cancer specific gene, which is highly expressed in advanced infiltrating breast carcinomas but not in normal or benign breast tissue. Aberrant expression of SNCG was also associated with ovary cancer progression. Synucleins are a family of small proteins consisting of 3 known members, α synuclein (SNCA), β synuclein (SNCB), and γ synuclein (SNCG). Synucleins has been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). However, studies also indicated the potential role of synucleins particularly SNCG in the pathogenesis of steroid-responsive tumors of breast and ovary. What role SNCG has in breast and ovary and how it is implicated in breast and ovary cancer remains a mystery. The association between SNCG expression and the progression of steroid dependent cancers of breast and ovary promoted us to investigate the role of SNCG in regulation of estrogen receptor ER-α.

BODY

A notable finding relevant to this study is that SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α in breast cancer cells. Since SNCG binds to ER-α, Hsp70, and Hsp90 in the absence of ligand but does not bind to ER-α and Hsp90 following ligand binding, these data suggest that SNCG is not likely to function as a coactivator involved in the post ligand binding events (such as DNA binding); but rather functions as chaperone to maintain ER-α compatible for high affinity ligand binding. We **hypotheses** that: 1) one of the critical functions of SNCG on breast cancer pathogenesis is to stimulate ER-α transcriptional activity; and 2) SNCG stimulates ER-α activation by participating in Hsp-based multiprotein chaperone system for efficient activation of steroid receptors; and this stimulation of ER-α activation is mediated at the stage of hormone binding.

SA1. To determine the mechanism of SNCG-stimulated ER-α activation; particularly we will focus on Hsp-based multiprotein chaperone complex for ER-α and to determine whether the SNCG-stimulated hormone dependent ER-α transactivation is mediated at the stage of hormone binding (Finished. Cancer Res 63: 3899-3903, 2003; Cancer Res 64: 4539-4546, 2004).

SA1-1. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells (Cancer Res 63: 3899-3903, 2003). Augmentation of SNCG expression stimulated transcriptional activity of ERα, whereas compromising SNCG expression suppressed ERα signaling. The SNCG-stimulated EFα signaling was demonstrated in three different cell systems:

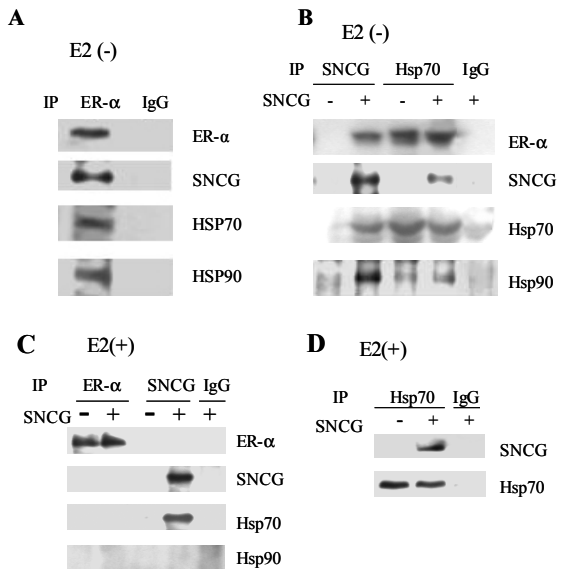
1. *Overexpression of SNCG stimulated transcriptional activity of ERα.* Transfection of SNCG gene into the SNCG-negative and ER-positive MCF-7 cells did not affect ERα expression but significantly stimulated E2-mediated activation of ERα. Overexpression of SNCG gene in MCF-7 cells increased E2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ERα was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ERα in the absence of E2.
2. *Co-transfection of SNCG and ERα into SNCG-negative and ERα-negative MDA-MB-435 cells*. Treatment of ER-α-transfected MDA-MB-435 cells with E2 activated reporter activity, indicating the functional transcriptional activity of the transfected ERα gene. A significant stimulation of ERα signaling by SNCG was observed in MDA-MB-435 cells when the cells

were co-transfected with ERα and SNCG constructs. SNCG increased ligand-dependent transcriptional activity 3.7-fold over the control cells.

3. *Antisense blocking SNCG expression in ERα-positive and SNCG-positive T47D cells*. The effect of SNCG expression on ERα transactivation was further demonstrated by inhibiting endogenous SNCG expression with SNCG antisense mRNA in T47D cells that express high levels of SNCG. Stable transfection of the SNCG antisense construct into T47D cells significantly reduced SNCG expression to 25% of that in control T47D cells. While E2 significantly stimulated the reporter activity in the control T47D cells, inhibition of SNCG expression reduced E2-responsive activity in two stable antisense-transfected T47D cell lines, AS-1 and AS-3 cells, to 21% and 13% of that in control T47D cells, respectively. Treatment of T47D cells with E2 resulted in a 25-fold increase over the non-treated cells. However, only 3.3- and 5.2-fold increase was observed in the AS-3 and AS-1 cells, respectively.

SA1-2. Mechanisms by which SNCG stimulates ER signaling and ligand-dependent growth (Cancer Res 64: 4539-4546, 2004). It is well documented that the activation of steroid receptors is modulated by a heterocomplex with several molecular chaperones, particularly with Hsp90 and Hsp70, which associate with the unliganded steroid receptors and maintain them in a high affinity hormone-binding conformation. The chaperone-like activity of synucleins has been demonstrated in the cell-free system. Because SNCG stimulated ligand-dependent transcriptional activity of ERα, which can be blocked by antiestrogen, we reason that SNCG may have a chaperone activity and participate in Hsp-based multiprotein chaperone complex for ERα.

SNCG is a novel Hsp70-associated chaperone, participated in the Hsp-based multiprotein chaperone complex for ERα. SNCG bound to the unliganded form of ER-α, Hsp90, and Hsp70. We investigated if SNCG can physically and functionally interact with ERα, Hsp70, and Hsp90 in SNCG transfected MCF-7 cells by co-immunoprecipitation assays. IP of ERα co-precipitated SNCG, Hsp70 and Hsp90 in the absence of estrogen (***Fig. 1A***) and *vice versa* (***Fig. 1B***), indicating that SNCG participated in a heterocomplex with Hsp90, Hsp70, and ER-α in the absence of estrogen. However, SNCG dissociated from ERα after the cells were treated with E2 (***Fig. 1C***). The binding pattern of SNCG to the unliganded ERα is same to that of Hsp90 and Hsp70, which only binds to the unliganded ER-α (34). Similar to its binding pattern to ERα, SNCG only bound to Hsp90 in the absence of estrogen (***Fig. 1B***). After cells were treated with E2, the liganded ER-α dissociated from SNCG, Hsp70, and Hsp90 (***Fig. 1C***). However, in contrast to its binding pattern to ERα and Hsp90, SNCG was found to bind to Hsp70 under the conditions both without (***Fig. 1B***) and with E2 (***Fig. 1D***), indicating that SNCG binds to Hsp70 constitutively regardless of whether Hsp70 is associated with ERα.



SNCG increases ligand binding by ERα. It has been proposed that Hsp-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding. We examined whether the SNCG-stimulated hormone dependent ERα transactivation results from increased estradiol binding by the receptor.

As demonstrated in **Fig. 2**, SNCG significantly enhanced ligand-binding by ER. The biggest increases in the ligand binding were observed at the lower concentration range of 0.0025-1 nM of ligand. At the concentration of 1 nM of E2, the ligand binding was increased 160% in the SNCG-positive cells vs. the SNCG-negative control cells. Scatchard analysis revealed two binding sites (**Fig. 3**). The high affinity state was apparent in a linear plot between 0.0025nM and 1 nM of estradiol, and the low affinity state between 1 nM and 10 nM of estradiol. SNCG overexpression significantly enhanced the high affinity state of ER in MCF-7 cells, resulting in a 2.3-fold increase in high affinity binding capacity. While the high affinity binding capacity in MCF-7-neo cells was saturated when the bound E2 reached at 0.06 pmol/mg protein, the high affinity binding capacity in MCF-7-SNCG cells was saturated when the bound E2 reached at 0.14 pmol/mg protein. These data indicate that SNCG affects ER-α signal transduction pathway at the step of ligand binding by increasing the number of high affinity ligand-binding sites.

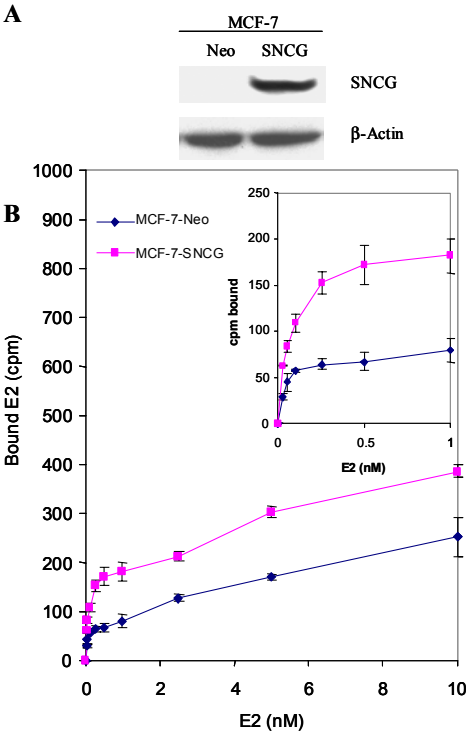


Fig. 2. Estrogen-binding capability for ER in SNCG-transfected MCF-7 cells (MCF-7-SNCG) and control neo-transfected cells (MCF-7-Neo). Cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. The transfected cells were enriched with Neomycin selection for 12 days before the hormone binding assay. **A**, Western blot analysis of SNCG expression in pooled SNCG-transfected MCF-7 cells after selection with G418. **B**. Titration of ^{3}H-E2 in MCF-7-Neo and MCF-7-SNCG cells. *Inset*, enlarged view of ^{3}H-E2 titration from 0-1 nM of ligand.

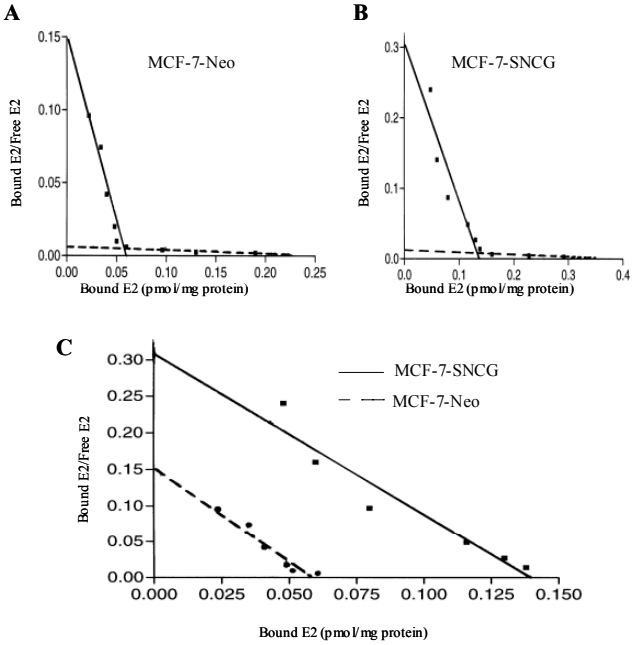


Fig. 3. Scatchard analysis of the ligand-binding by ER from MCF-7-Neo cells (**A**) and MCF-7-SNCG cells (**B**). *Unbroken line*, high affinity sites; *broken line*, low affinity sites. **C,** The high affinity sites of MCF-7-Neo and MCF-7-SNCG cells. Specific binding was determined by subtracting the non-specific binding from samples incubated with 100-fold excess of non-labeled E2. Each data point is the mean ± SD of triplicate samples.

SA2. To study the biological relevance of SNCG-stimulated ER-α signaling to hormone-dependent tumorigenesis. The major goal of this specific aim is to determine if SNCG expression

in breast cancer cells stimulate estrogen-mediated tumor growth. We will determine if expression of SNCG in breast cancer cells will stimulate estrogen-mediated tumor growth in nude mice. This will be investigated in MCF-7 and T47D cells.

SA2-1. Stimulation of estrogen-mediated cell growth. The SNCG-stimulated ERα transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. It has been demonstrated in different systems that the interaction between SNCG and ERα stimulated cell growth.

MCF-7 cells. The cellular proliferation of two stably SNCG-transfected MCF-7 cell clones were compared with that of SNCG-negative neo clones. ***Fig 4A*** shows that while SNCG had no significant effect on the proliferation of SNCG-transfected cells compared to MCF-neo cells in the absence of E2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E2 stimulated an average cell proliferation 2.4-fold over controls. However, E2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation vs. controls, suggesting that SNCG expression renders the cells more responsive to E2-stimulated cell proliferation.

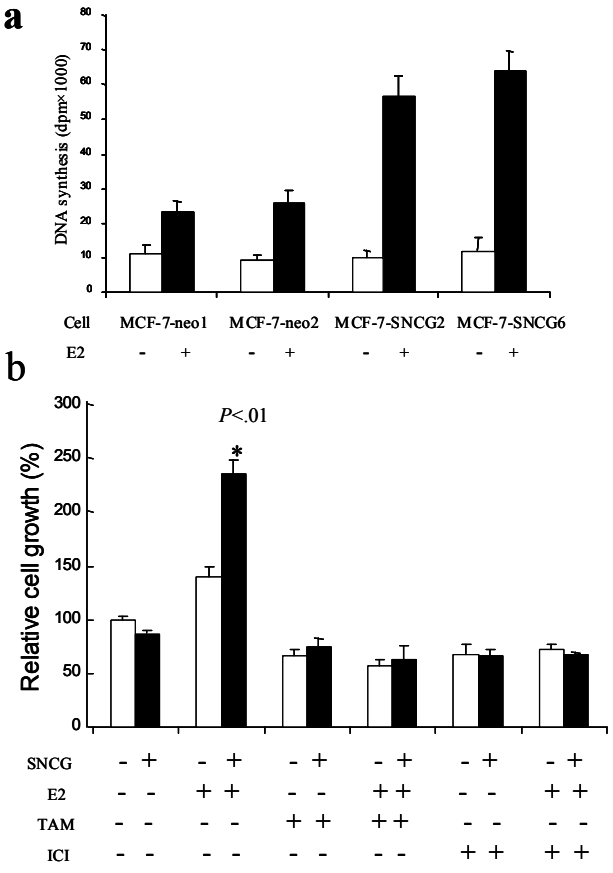


Fig. 4. SNCG stimulated ligand-dependent cell proliferation. For all experiments, cells were cultured and synchronized in the ligand-free Conditioned Cell Culture medium for 4 days before the hormone treatments. **A,** Stimulation of cell proliferation by SNCG overexpression. Cells were treated with or without 1 nM E2 for 24 hours. Cell proliferation was measured by ^{3}H- thymidine incorporation. Data are means ± SD of three cultures. **B**, Effect of antiestrogens on SNCG-stimulated cell growth. Cells were treated with or without 1 nM of E2, 1 μM of tamoxifen, or 1 μM of ICI for 6 days before harvesting. Media were changed every two days with fresh estrogen and antiestrogens. Cell growth was measured using a cell proliferation kit (XTT). Data are the mean ± SD of quadruplicate cultures. Open bar represents MCF-neo1 cells; closed bar represents MCF-SNCG6 cells.

Blocking endogenous SNCG in T47D cells by antisense construct. Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing SNCG antisense mRNA was significantly suppressed. When cells were cultured in soft agar without E2, there were very few colonies formed in both T47D group and T47D-SNCG antisense group. Treatment of T47D cells with E2 resulted in a 19-fold increase of colonies over the non-treated cells. However, treatment of T47D cells expressing SNCG antisense mRNA with E2 resulted in only 3-fold increase over the non-treated cells (***Fig. 5***). These data demonstrated that inhibition of endogenous SNCG expression dramatically diminished the cell growth in response to estrogen.

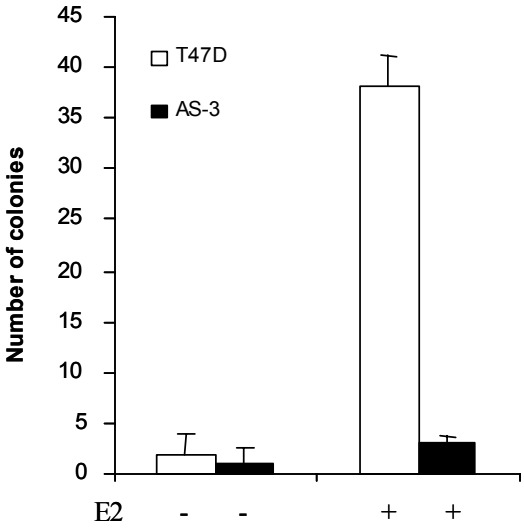


Fig. 5. Effect of inhibiting endogenous SNCG expression on soft agar colonies formation capability of T47D cells. T47D and SNCG antisense stably transfected AS-3 clone were cultured into the top layer soft agar and treated with or without 1 nM of E2 as described in Materials and Methods. The number of colonies was counted after 2 weeks of plating using a Nikon microscope at 100× amplification. Triplicate wells were assayed for each condition.

SA2-2. Stimulation of estrogen-dependent mammary tumorigenesis by SNCG (Cancer Res 64: 4539-4546, 2004). An orthotopic nude mouse model was used to study the effects of SNCG on tumor growth. Two independent experiments under different conditions were done to determine the effects of SNCG on mammary tumorigenesis.

We first analyzed the tumorigenesis in response to E2 in the non-ovariectomized intact mice. It was previously demonstrated that the circulating E2 level in the non-ovariectomized mice is 26 pg/ml (31), which compares to the low levels found in postmenopausal women (32). To override the endogenous levels of estrogen, all the mice were supplemented with E2 (0.72 mg/pellet) one day before injection of 5 x10^6 cells. After a lag phase of 8-10 days, 29 of 32 (91%) injections in the mice given implants of SNCG positive MCF-SNCG2 and MCF-SNCG6 cells developed tumors. In contrast, only 21 of 32 (66%) injections in the mice given implants of SNCG negative MCF-neo1 and MCF-neo2 cells developed tumors (***Table 1***).

Table 1. Effects of SNCG expression on tumor incidence and tumor growth of MCF-7 cells

		E2	*Tumor incidence*	*Tumor volume (mm^3)*	
Experiment	Group		tumor/total (%)	Day 21	Day 35
	MCF-SNCG2	+	14/16 (88)	234 ± 69	1850 ± 280
	MCF-SNCG6	+	15/16 (94)	351 ± 78	2521 ± 390
	MCF-neo1	+	10/16 (63)	101 ± 39	491 ± 92

Cells were injected into the mammary fat pads, and tumor volumes and tumor incidence were determined as described in Materials and Methods. Each mouse received two injections. Tumor volumes were measured at 21 days and 35 days following cell injection and are expressed as means ± SEs (number of tumors assayed). All the non-ovariectomized mice received an estrogen implantation one day before the cell injection. There were total 16 injections for 8 mice in each group, and each injection had 5 x 10^6 cells. Statistical comparisons for pooled SNCG positive clones relative to pooled SNCG negative clones indicated $P < 0.01$ for the mean tumor sizes and $P < 0.05$ for the tumor incidence. Statistical comparison for primary tumors was

analyzed by Student's *t* test. A chi-square test was used for statistical analysis of tumor incidence.

The tumor growths in MCF-SNCG clones were significantly stimulated. At 35 days following tumor cell injection, the size of MCF-SNCG6 tumors, which expressed relative high level of SNCG mRNA, was 4.8-fold of that in parental MCF-neo2 tumors and 3.7-fold of that in MCF-neo1 tumors. In addition, the tumor incidence was also increased. With 16 injections, while 15 implants in MCF-SNCG6 cells developed tumors, only 10 implants from MCF-neo1 and 11 implants from MCF-neo2 developed tumors, respectively. The tumor growth of MCF-SNCG2 cells was also significantly stimulated, with 3.5-fold and 2.7-fold increase in tumor size as compared to MCF-neo2 and MCF-neo1 tumors, respectively. ***Fig. 6*** shows growth kinetics. After a slow growth phase of 14 days, tumor growth of MCF-SNCG2 and MCF-SNCG6 clones were significantly enhanced as compared to that of MCF-neo1 and MCF-neo2 clones. Thus, the tumorigenesis of MCF-7 cells in response to E2 was significantly stimulated by the SNCG overexpression.

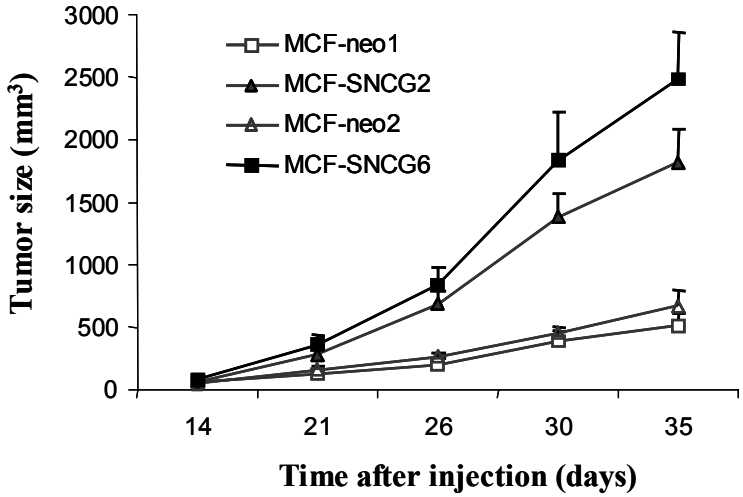


Fig. 6. Stimulation of MCF-7 tumor growth by SNCG. Each of the eight E2-supplemented non-ovariectomized mice in each group received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each clone at each time point. Each point represents the mean of tumors ± SE (bars).

We then analyzed the requirement of E2 for SNCG-mediated tumor stimulation under more stringent conditions including use of ovariectomized mice and reduction of injected tumor cells from 5 x 10^6 to 1.5 x 10^6. As shown in ***Table 2***, when the number of injected MCF-neo1cells was reduced, the tumor incidence was greatly reduced from 63% to 0% both in the absence and presence of E2 supplement for up to 4 weeks, indicating a non-tumorigenic condition for SNCG-negative MCF-7 cells even with estrogen stimulation for 4 weeks. At the week 7, a 30% of tumor incidence with small tumor size (44 mm^3) was observed. However, for MCF-SNCG6 cells, although a same non-tumorigenic phenotype was observed under the conditions with reduced cell number and in the absence of E2, when E2 was supplemented, the tumor incidence reached to 90% at week 3 after cell inoculation, which is similar to 94% of tumor incidence in the experiment with higher injected cell numbers (***Table 1***). Furthermore, the tumor size of SNCG-positive cells is 4.4 fold over that of SNCG-negative cells. These data indicate that estrogen is necessary for SNCG-mediated tumor stimulation in the xenograft model.

Table 2. Stimulation of estrogen-mediated tumorigenesis by SNCG

Group	E2	Tumor Incidence (%)							Tumor Vol (mm^3)
	Week	1	2	3	4	5	6	7	Week 7
MCF-neo1	-	0	0	0	0	0	0	0	
MCF-neo1	+	0	0	0	0	20	30	30	44±10
MCF-SNCG6	-	0	0	0	0	0	0	0	
MCF-SNCG6	+	40	80	90	90	90	90	90	194±35

Ovariectomized mice were treated with or without E2 pellet. There were total 10 injections for 5 mice in each group, and each injection had 1.5 x 10^6 cells. Only measurable tumors were used to calculate the mean tumor volume. Statistical comparisons for SNCG positive clone relative to SNCG negative clone indicated $P < 0.001$ for both tumor incidence and mean tumor sizes in the presence of E2.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

1. SNCG strongly stimulated the ligand-dependent transcriptional activity of ERα in breast cancer cells. Augmentation of SNCG expression stimulated transcriptional activity of ERα, whereas compromising SNCG expression suppressed ERα signaling.
2. Stimulation of estrogen-mediated cell growth. The SNCG-stimulated ERα transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth.
3. SNCG-mediated stimulation of ERα signaling and cell growth can be inhibited by antiestrogens.
4. Stimulation of estrogen-dependent mammary tumorigenesis by SNCG.
5. SNCG is a novel Hsp70-associated chaperone**,** participated in the Hsp-based multiprotein chaperone complex for ERα, and increased ligand binding by ERα.

CONCLUSIONS

Although synucleins are emerging as central players in the formation of pathologically insoluble deposits characteristic of neurodegenerative diseases, γ Synuclein (SNCG), previously identified as a breast cancer specific gene (BCSG1), is also highly associated with breast or ovarian cancer progression. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated a chaperone activity of SNCG in the heat shock protein-based multiprotein chaperone complex for stimulation of ER-α signaling. As an ER-α-associated chaperone, SNCG participated in Hsp-ER-α complex, enhanced the high affinity ligand-binding capacity of ER-α, and stimulated ligand-dependent activation of ER-α. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of mammary tumorigenesis in response to estrogen. These data indicate that SNCG is a new chaperone protein in the Hsp-based multiprotein chaperone complex for stimulation of ligand-dependent ER-α signaling and, thus, stimulates hormone responsive mammary tumorigenesis.

[CANCER RESEARCH 64, 4539–4546, July 1, 2004]

γ Synuclein, a Novel Heat-Shock Protein-Associated Chaperone, Stimulates Ligand-Dependent Estrogen Receptor α Signaling and Mammary Tumorigenesis

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ABSTRACT

Synucleins are emerging as central players in the formation of pathologically insoluble deposits characteristic of neurodegenerative diseases. γ *synuclein* (*SNCG*), previously identified as a breast cancer-specific gene (*BCSG1*), is also highly associated with breast or ovarian cancer progression. However, the molecular targets of SNCG aberrant expression in breast cancer have not been identified. Here, we demonstrated a chaperone activity of SNCG in the heat-shock protein (Hsp)-based multiprotein chaperone complex for stimulation of estrogen receptor (ER)-α signaling. As an ER-α-associated chaperone, SNCG participated in Hsp-ER-α complex, enhanced the high-affinity ligand-binding capacity of ER-α, and stimulated ligand-dependent activation of ER-α. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of mammary tumorigenesis in response to estrogen. These data indicate that SNCG is a new chaperone protein in the Hsp-based multiprotein chaperone complex for stimulation of ligand-dependent ER-α signaling and thus stimulates hormone-responsive mammary tumorigenesis.

INTRODUCTION

Using the differential cDNA sequencing (1–3), we undertook a search for differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer-specific gene, *BCSG1*, was identified as a putative breast cancer marker (1). This gene was highly expressed in the advanced breast cancer cDNA library but scarce in a normal breast cDNA library. BCSG1 is not homologous to any other known growth factors or oncogenes. Rather, there is extensive sequence homology to the neural protein synuclein. Subsequent to the isolation of *BCSG1*, γ *synuclein* (*SNCG*; Ref. 4) and *persyn* (5) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be identical to BCSG1. Thus, the previously identified BCSG1 has also been named as SNCG and is considered to be the third member of the synuclein family (6).

Synucleins are a family of small proteins consisting of 3 known members, α synuclein (SNCA), β synuclein (SNCB), and γ synuclein (SNCG). Synucleins have been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in *SNCA* is genetically linked to several independent familial cases of PD (7). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (8, 9). SNCA peptide known as nonamyloid component of plaques has been implicated in amyloidogenesis in AD (10, 11). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (12, 13). Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, they have also been implicated in nonneural diseases, particularly in the hormone-responsive cancers of breast and ovary (1, 4, 14–22).

Being identified as a breast cancer-specific gene, *SNCG* expression in breast follows a stage-specific manner (1). Overall *SNCG* mRNA expression was detectable in 39% of breast cancers. However, 79% of stage III/IV breast cancers were positive for SNCG expression, whereas only 15% of stage I/II breast cancers were positive for SNCG expression. In contrast, the expression of SNCG was undetectable in all benign breast lesions (17). The expression of SNCG was strongly correlated with the stage of breast cancer. Overexpression of SNCG in breast cancer cells led to a significant increase in cell motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo* (14). Overexpression of synucleins, especially SNCG and SNCB, also correlated with ovarian cancer development (4, 19). Although synucleins' (α, β, and γ) expression was not detectable in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all three synucleins (α, β, and γ) simultaneously (19). The involvement of SNCG in hormone-responsive cancers of breast and ovary promoted us to explore the potential role of SNCG in cellular response to estrogen. Previously, we investigated the functions of SNCG in regulating estrogen receptor transcriptional activity and demonstrated that SNCG strongly stimulated the ligand-dependent transcriptional activity of estrogen receptor (ER)-α in breast cancer cells. Augmentation of SNCG expression stimulated the transcriptional activity of ER-α, whereas compromising endogenous SNCG expression suppressed ER-α signaling (16). In the present study, we evaluated the mechanism by which SNCG stimulated ER-α transcriptional activity and its biological relevance to estrogen-stimulated mammary tumorigenesis. The results indicated that SNCG stimulates ER-α signaling by acting as a chaperone in the heat-shock protein (Hsp)-based multiprotein chaperone complex of ER-α and enhances its high-affinity ligand binding.

MATERIALS AND METHODS

Reagents. Improved MEM, FCS, and charcoal-stripped FCS were obtained from Biosource International (Camarillo, CA). 17-β-Estradiol (E_2) and geldanamycin (GA) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Estradiol was from Perkin-Elmer Life Science, Inc. (Boston, MA). The mammalian expression plasmid pCI-neo was from Promega (Madison, WI).

Cell Culture. All cell lines used in this study (MCF-7, T47-D) were originally obtained from the American Type Culture Collection. Proliferating subconfluent human breast cancer cells were harvested and cultured in the phenol red-free improved MEM containing 5% charcoal-stripped FCS for 4 days before addition of indicated dose of E_2. Cells in the absence or presence of E_2 were collected for ligand-binding assay or immunoprecipitation/Western blot analyses.

Gene Transfection. Subconfluent cells in 12-well plate were incubated with 2 μg of expression vectors in 1 ml of serum-free improved MEM containing LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) for 5 h. Culture was washed to remove the excess vector and LipofectAMINE and then postincubated for 24 h in fresh culture medium to allow the expression of transfected gene. For stable transfection, transfected cells were routinely

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selected with G418 (600 μg/ml). Individual colonies were picked to establish stable clones.

Immunoprecipitation. Cells were cultured in 100-mm cell culture dishes in ligand-free medium for 4 days as described in "Cell Culture." Cells were treated with or without E_2 for 3 days before the total cell lysates were prepared. Cells were lysed in solution containing 1× PBS, 1% Triton X-100, 10 mM sodium molybdate, 2 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cells then were disrupted by sonication and centrifuged for 10 min at 10,000 × *g*. The protein concentrations of the supernatant were determined by BCA Protein Assay kit (Pierce). Cell lysates (1 mg of total cellular protein) were incubated with 2 μg of indicated antibody at room temperature for 1.5 h followed by the addition of protein G-Sepharose. The beads were washed four times with the lysis buffer described above, and the bound proteins were eluted with 1× SDS gel-loading buffer followed by Western blotting.

Western Blot Analysis. Proteins were fractionated by electrophoresis through a SDS polyacrylamide gel, and the proteins were then transferred onto a nitrocellulose membrane. The membrane was blocked for 2 h in blocking buffer and then incubated with primary antibodies at room temperature for 2 h. After washing with Tris-buffered saline/0.2% Tween 20 buffer, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase, and the protein was detected using chemiluminescence method followed by autoradiography. Antibodies used for immunoprecipitations and Western blot analyses were as follows: anti-γ-synuclein antibody (goat polyclonal antibody E-20, 1:300 dilution); anti-ER-α antibody (rabbit polyclonal antibody HC-20, 1:300 dilution); anti-Hsp-70 antibody (goat polyclonal antibody sc-1060; 1:1000 dilution); anti-heat shock cognate 70 antibody (goat polyclonal antibody, 1:1000 dilution); anti-Hsp-90 antibody (rabbit polyclonal antibody sc-7947; 1:1000 dilution); normal goat IgG (sc-2028); normal rabbit IgG (sc-2027); and anti-actin antibody (goat polyclonal antibody sc-1615). These antibodies were from Santa Cruz Biotechnology. Anti-phospho-estrogen receptor α (Ser^{167}; 1:500 dilution), anti-phospho-Akt (Ser^{473}; 1:500 dilution) and anti-Akt (1:1000 dilution) are from Cell Signaling Technology (Beverly, MA).

Ligand-Binding Assay. MCF-7 cells were plated into 24-well plate at 50,000 cells/well and cultured in estrogen-free medium for 3 days before the binding assay. E_2 binding by ER was assayed by measuring the bound [^{3}H]estradiol following a 1.5-h incubation with different concentrations of [^{3}H]estradiol as follows: 0, 0.0025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM. Cells were then washed three times with 0.5 ml of PBS and dissolved with 0.1 ml of 0.1 N NaOH. The cell lysate was transferred into a 1.5-ml Eppendorf tube and neutralized with 0.1 ml of 0.1 M NaAc. Aliquots of 150 μl were counted in a liquid scintillation counter. Counts from samples, which were incubated with a 100-fold excess of unlabeled E_2 (nonspecific binding), were subtracted from the total counts to give the values for specific ligand binding. Triplicate wells were assayed for each condition. Each value was normalized against the protein concentration.

Assays for the Transcriptional Activity of ER-β. Cells were transiently transfected with a firefly luciferase reporter construct (pERE4-Luc) containing four copies of the estrogen response element (ERE). For the cotransfection experiments, the plasmid DNA ratio of pERE4Luc to expression vectors of ER-β or SNCG was 2:1. A Renilla luciferase reporter, pRL-SV40-Luc, was used as an internal control for transfection efficiency. Luciferase activities in total cell lysate were measured using the Promega Dual Luciferase Assay System. Absolute ERE promoter firefly luciferase activity was normalized against Renilla luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed.

Tumor Growth in Athymic Nude Mice. A nude mouse tumorigenesis assay was performed as we described previously (14). Briefly, estrogen pellets (0.72 mg/pellet; Innovative Research of America, Toledo, OH) were implanted s.c. in all athymic nude mice (experiment 1) and in some ovariectomized athymic nude mice (experiment 2; Frederick Cancer Research and Development Center, Frederick, MD). Approximately 5×10^6 cells (experiment 1) or 1.5×10^6 cells (experiment 2) were injected into a 6-week old female athymic nude mouse. Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined at weekly intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point.

RESULTS

SNCG Participated in the Hsp-Based Multiprotein Chaperone Complex for ER-α. It is well documented that the activation of steroid receptors is modulated by a heterocomplex with several molecular chaperones, particularly with Hsp90 and Hsp70, which associate with the unliganded steroid receptors and maintain them in a high-affinity hormone-binding conformation (23–26). The chaperone-like activity of synucleins has been demonstrated in the cell-free system by monitoring the aggregation of thermally denatured proteins (27). Because SNCG stimulated ligand-dependent transcriptional activity of ER-α, which can be blocked by antiestrogen (16), we reason that SNCG may have a chaperone activity and participate in Hsp-based multiprotein chaperone complex for ER-α. In this regard, we investigated if SNCG can physically and functionally interact with ER-α, Hsp70, and Hsp90 in SNCG-transfected MCF-7 cells by co-immunoprecipitation assays. Immunoprecipitation of ER-α coprecipitated SNCG, Hsp70, and Hsp90 in the absence of estrogen (Fig. 1*A*) and *vice versa* (Fig. 1*B*), indicating that SNCG participated in a heterocomplex with Hsp90, Hsp70, and ER-α in the absence of estrogen. However, SNCG dissociated from ER-α after cells were treated with E_2 (Fig. 1*C*). The binding pattern of SNCG to the unliganded ER-α is same to that of Hsp90 and Hsp70, which only bind to the unliganded ER-α (24). Similar to its binding pattern to ER-α, SNCG only bound to Hsp90 in the absence of estrogen (Fig. 1*B*). After cells were treated with E_2, the liganded ER-α dissociated from SNCG, Hsp70, and Hsp90 (Fig. 1*C*). However, in contrast to its binding pattern to ER-α and Hsp90, SNCG was found to bind to Hsp70 under the conditions both without (Fig. 1*B*) and with E_2 (Fig. 1*D*), indicating that SNCG binds to Hsp70 constitutively regardless of whether Hsp70 is associated with ER-α.

We also investigated the interactions among endogenous SNCG,

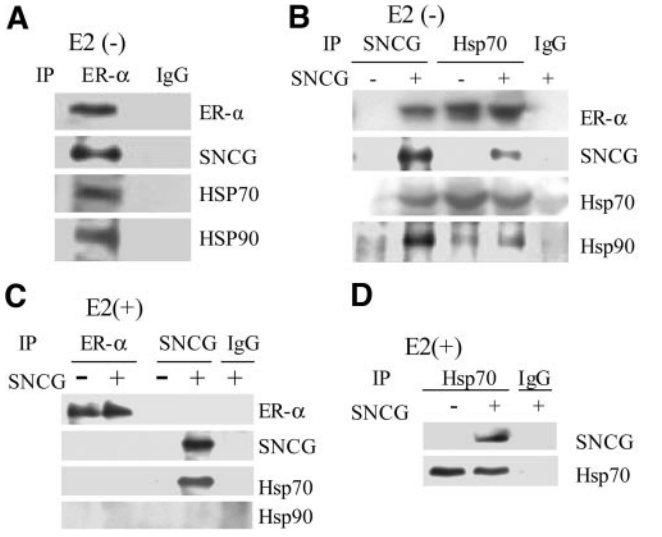

Fig. 1. Interaction between γ synuclein (SNCG) and estrogen receptor (ER)-α, heat-shock protein (Hsp)70, and Hsp90 in SNCG-transfected MCF-7 cells. Cells were transiently transfected with pCI-SNCG or the control vector pCI-neo and then selected with G418 as we described previously (16). *A* and *B*, association of SNCG with endogenous ER-α, Hsp70, and Hsp90 in the absence of estradiol (E_2). Total cell lysates were isolated from the cells cultured in the E_2-free conditioned medium, and equal amounts of protein were subjected to immunoprecipitation (IP) with different antibodies. IP with anti-ER (*A*), anti-SNCG (*B*), anti-Hsp70 antibodies (*B*), and control rabbit (*A*) or goat IgG (*B*) followed by Western blotting for ER-α, SNCG, Hsp70, and Hsp90. *C* and *D*, association of SNCG with Hsp70 but not ER-α and Hsp90 in the presence of E_2. Total cell lysates were isolated from the cells cultured in the conditioned medium containing 10 nM E_2, and equal amounts of protein were subjected to IP with different antibodies. *C*, IP with anti-ER-α, anti-SNCG antibodies, and control goat IgG followed by Western blot for ER-α, SNCG, Hsp70, and Hsp90. *D*, IP with anti-Hsp70 antibody and control goat IgG followed by Western blotting for SNCG and Hsp70.

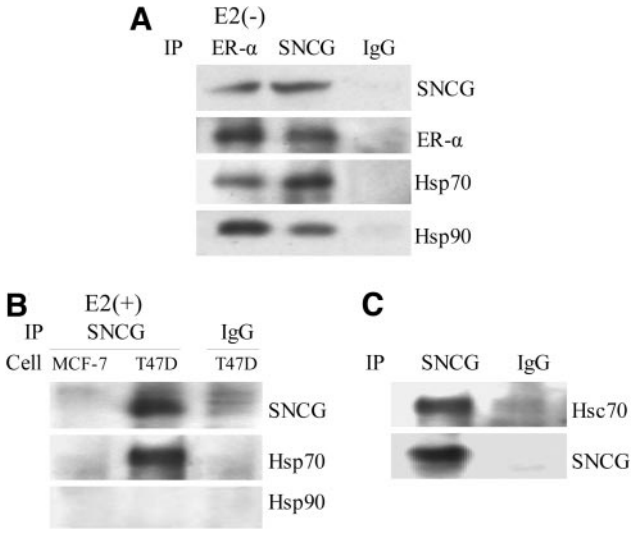

Fig. 2. Interaction between endogenous γ synuclein (SNCG), estrogen receptor (ER)-α, heat-shock protein (Hsp)70, heat-shock cognate (Hsc)70, and Hsp90 in T47D cells. *A*, association of endogenous SNCG with ER-α, Hsp70, and Hsp90 in the absence of estradiol (E_2). Total cell lysates were isolated from the cells cultured in the E_2-free conditioned medium, and equal amounts of protein were subjected to immunoprecipitation (IP) with anti-ER-α, anti-SNCG antibodies, and normal IgG. The immunoprecipitates were analyzed for Hsp70, SNCG, Hsp90, and ER-α after Western blotting using anti-Hsp70, anti-SNCG, anti-Hsp90, and anti-ER-α antibodies. *B*, association of endogenous SNCG with Hsp70 in the presence of E_2. Total cell lysates were isolated from the cells cultured in the conditioned medium containing 10 nM E_2, and equal amounts of protein were subjected to IP with anti-SNCG antibody and normal goat IgG. The nontransfected MCF-7 cell lysates were also subjected to IP as a negative control for SNCG IP. The immunoprecipitates were subjected to Western blotting using antibodies against Hsp70, SNCG. *C*, association of endogenous SNCG with Hsc70. Lysates from T47-D cells were subjected to IP with anti-SNCG antibody and normal goat IgG. The immunoprecipitates were subjected to Western blotting using antibodies against Hsc70, SNCG.

ER-α and Hsp. T47D cells express both SNCG and ER-α and therefore were chosen to study the interaction between SNCG and ER-α and Hsp in the physiological situation. Same interaction pattern between endogenous SNCG and ER-α and Hsp was observed in T47D cells as that we demonstrated in SNCG transfected MCF-7 cells (Fig. 2). When T47D cells were cultured under the estrogen-free conditions, endogenous SNCG was coimmunoprecipitated with ER-α, Hsp70, and Hsp90 (Fig. 2A). However, after the treatment of E2, SNCG dissociated from ER-α and Hsp90 but still bound to Hsp70 (Fig. 2*B*), indicating that the interaction between the endogenous SNCG, ER-α, Hsp70, and Hsp90 proteins also occurs in the physiological situation. In addition, SNCG also bound to heat shock cognate 70, the cognate form of Hsp70 (Fig. 2*C*).

SNCG Enhances Ligand Binding by ER. It has been proposed that Hsp90-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding (24). We examined whether the SNCG-stimulated hormone-dependent ER-α transactivation results from increased E_2 binding by the receptor. This was investigated by detecting the hormone-binding activity of ER using a ligand-binding assay in MCF-7 cells. In this experiment, growing cell cultures were treated with [^{3}H]labeled E_2 over a range of hormone concentrations. As shown in Fig. 3, SNCG significantly enhanced ligand binding by ER. The biggest increases in the ligand binding were observed at the lower concentration range of 0.0025–1 nM ligand. At the concentration of 1 nM E_2, the ligand binding was increased 160% in the SNCG-positive cells *versus* the SNCG-negative control cells. Although the levels of SNCG-enhanced ligand binding were gradually decreased with the increasing concentrations of ligand, the stimulated ligand binding was still considerably significant. At the concentration of 10 nM E_2, SNCG overexpression led to a 50% increase in the ligand binding. However, no difference in ligand binding was observed between MCF-7-neo and MCF-7-SNCG cells at 100 nM E_2 (data not shown). These data suggest that SNCG enhances ER's ability to respond to low levels of ligand.

The binding data are complex because there are two binding states that differed in their affinity for the hormone. As revealed by Scatchard analysis (Fig. 4), the cutoff value for estrogen concentration to differentiate high- from low-affinity binding is 1 nM. The high-affinity state was apparent in a linear plot between 0.0025 and 1 nM E_2, and the low-affinity state between 1 and 10 nM E_2. The existence of both high- and low-affinity states of steroid receptors have been observed previously (28). To more closely examine the effect of SNCG on high-affinity ligand binding, data in Fig. 4*C* shows the high-affinity binding state in MCF-7-neo and MCF-7-SNCG cells. SNCG overexpression significantly enhanced the high-affinity state of ER in MCF-7 cells, resulting in a 2.3-fold increase in high-affinity binding capacity. Although the high-affinity binding capacity in MCF-7-neo cells was saturated when the bound E_2 reached 0.06 pmol/mg protein, the high-affinity binding capacity in MCF-7-SNCG cells was saturated when the bound E_2 reached 0.14 pmol/mg protein. These data indicate that SNCG affects ER-α signal transduction pathway at the step of ligand binding by increasing the number of high-affinity ligand-binding sites.

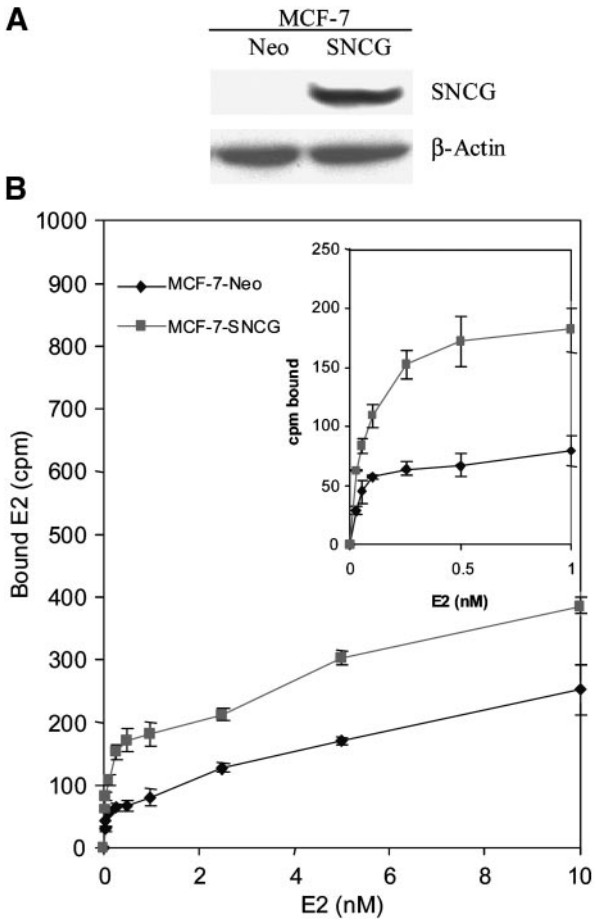

Fig. 3. Estrogen-binding capability for estrogen receptor in γ synuclein (SNCG)-transfected MCF-7 cells (MCF-7-SNCG) and control neo-transfected cells (MCF-7-Neo). Cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. The transfected cells were enriched with Neomycin selection for 12 days before the hormone-binding assay. *A*, Western blot analysis of SNCG expression in pooled SNCG-transfected MCF-7 cells after selection with G418. *B*, titration of [^{3}H]estradiol (E_2) in MCF-7-Neo and MCF-7-SNCG cells. *Inset*, enlarged view of [^{3}H]E_2 titration from 0 to 1 nM of ligand.

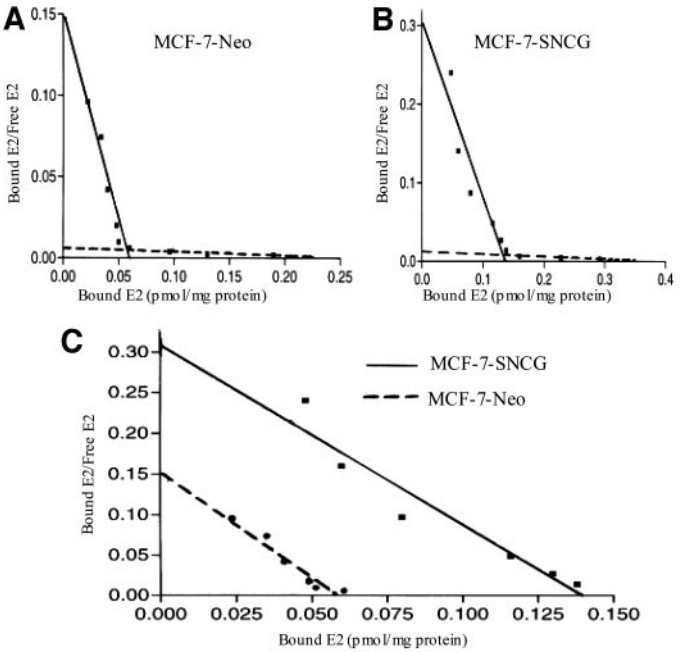

Fig. 4. Scatchard analysis of the ligand binding by estrogen receptor from MCF-7-Neo cells (*A*) and MCF-7-SNCG cells (*B*). *Solid line*, high-affinity sites; *dashed line*, low-affinity sites. *C*, the high-affinity sites of MCF-7-Neo and MCF-7-SNCG cells. Specific binding was determined by subtracting the nonspecific binding from samples incubated with 100-fold excess of nonlabeled estradiol (E_2). Each data point is the mean ± SD of triplicate samples.

Hsp90-Specific Compound GA Abolished the Stimulatory Effect of SNCG on ER-α Activity. To address whether the stimulatory effect of SNCG on ER-α ligand-binding activity is mediated by Hsp chaperone activity, we investigated the effect of the Hsp90 inhibitor GA on the ligand-binding activity of ER-α in MCF-7-neo cells and MCF-7-SNCG cells. GA inhibits the ATPase activity of Hsp90. We first analyzed the effect of GA treatment on ER-α levels. As demonstrated in Fig. 5*A*, transfection of SNCG gene into the SNCG-negative MCF-7 cells did not affect ER-α expression under the conditions with or without GA treatment. In the absence of GA, the basal levels of ER-α in control and SNCG-transfected cells are the same. Although treatment of the control cells with GA resulted in a decrease in ER-α levels, overexpression of SNCG did not affect GA-mediated degradation of ER-α. We next investigated the effect of GA on the ER-α ligand-binding activity in MCF-7-neo and MCF-7-SNCG cells. In the absence of GA, SNCG increased the ligand-binding capability of ER-α by 91%. Upon the treatment with GA, although considerable amounts of ER-α protein were still present in both MCF-7-neo and MCF-7-SNCG cells, the ligand-binding activity of ER-α in both cells was abolished (Fig. 5*B*). This data indicates that the stimulatory effect of SNCG on ER-α activity is Hsp dependent.

SNCG Did Not Affect Phosphorylation of ER-α. To additionally determine the molecular mechanisms for SNCG-stimulated ER-α transcriptional activity, we also studied if SNCG regulates ligand-dependent or ligand-independent phosphorylation of ER-α. Although E_2 directly binds to and activates ER, thereby enhancing estrogen-responsive genes transcription, ER-α can also be activated by ligand-independent phosphorylation, which is mediated by cytoplasmic proteins and signaling pathways such as mitogen-activated protein kinase- and Akt-mediated phosphorylation (29). Many growth factors such as epidermal growth factor can also activate ER-α by such cytoplasmic signaling pathways. As expected, treatment of MCF-7 cells with E_2 resulted in decreased levels of ER-α and stimulated Ser^{167} phosphorylation of ER-α. However, transfection of SNCG to MCF-7 cells didn't enhance Ser^{167} phosphorylation of ER-α in response to E_2 compared with mock-transfected cells (Fig. 6*A*). There was also no significant difference in Ser^{104} or Ser^{118} phosphorylation of ER-α (data not shown). In addition, SNCG overexpression in MCF-7 cells didn't enhance the phosphorylation of Akt in response to both E_2 and epidermal growth factor (Fig. 6*B*).

Effect of SNCG on Transcriptional Activity of ER-β. In an effort to address whether SNCG has general effect on other steroid hormone receptors relevant in breast cancer, we investigated the effect of SNCG on the transcriptional activity of ER-β. Because both ER-α and ER-β bind to the same EREs, we cotransfected ER-α-negative MDA-MB-435 cells with ER-β and ER reporter ERE4-luciferase (Fig. 7). In contrast to the previously demonstrated stimulation of ER-α signaling (16), SNCG did not stimulate the transactivation of ER-β. When cells were treated with E_2, a 15.7- and 13.5-fold increase relative to basal levels in ER-β reporter activity was observed in SNCG-negative and SNCG-positive cells, respectively. Therefore, expression of SNCG in breast cancer cells stimulates the transcriptional activity of ER-α but not ER-β, indicating that SNCG may not have a general effect on stimulation of transcriptional activity of steroid receptors.

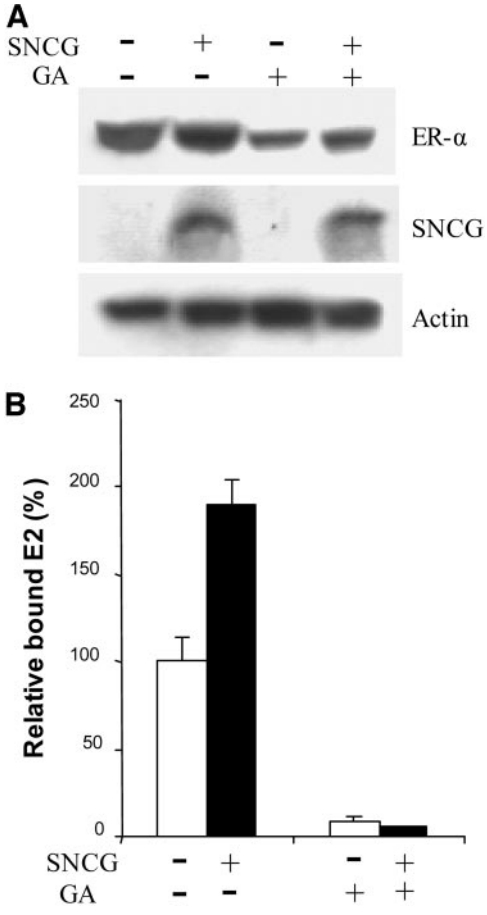

Fig. 5. Effect of heat-shock protein (Hsp)90 inhibitor geldanamycin (GA) on estrogen-binding capability of estrogen receptor (ER) in MCF-7 cells. *A*, Western blot analysis of ER and γ synuclein (SNCG) levels in pooled MCF-7-Neo and MCF-7-SNCG cells treated with or without GA. The pooled MCF-7-Neo and MCF-7-SNCG cells were cultured in the presence of 0.1 nM estradiol (E_2) and treated with or without 0.25 μM GA for 24 h. Cell lysates were normalized and subjected to Western blot analysis using the antibody against ER-α, SNCG, and actin, respectively. *B*, GA abolished the stimulatory effect of SNCG on estrogen-binding capability in MCF-7 cells. MCF-7-Neo and MCF-7-SNCG cells were treated with or without 0.25 μM GA for 24 h followed by the ligand binding assay with 0.1 nM [^{3}H]E_2. The data were presented as the percentage of the nontreated MCF-7-Neo controls, which was taken as 100%. Each data represent the mean ± SD of triplicate cultures.

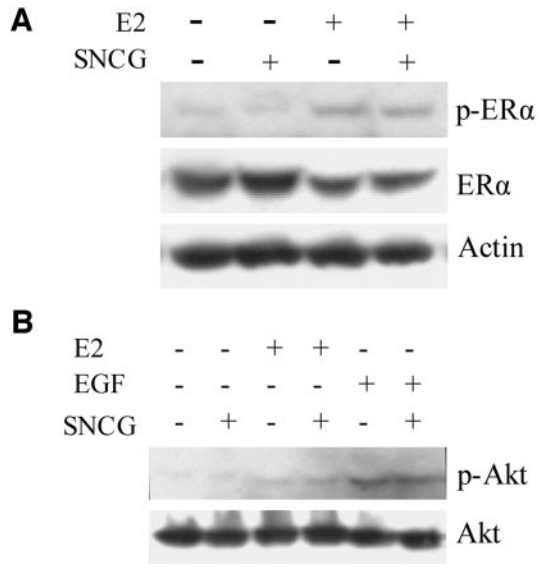

Fig. 6. Effect of γ synuclein (SNCG) overexpression on ER-α and AKT phosphorylation. *A*, MCF-7 cells were transiently transfected with pCI-SNCG or pCI-neo plasmids. After G418 selection for 12 days, the pooled population of transfected cells were treated with or without 10^{-9} M estradiol (E_2) or 50 ng/ml epidermal growth factor (EGF). Cell lysates were subjected to Western blot analysis using the antibodies against human Ser^{167}-phosphorylated ER-α, ER-α, and actin, respectively. SNCG overexpression didn't affect E_2 and EGF-induced ER-α phosphorylation. *B*, the pooled population of SNCG-transfected cells and mock-transfected cells were treated with or without 10^{-9} M E_2 or 50 ng/ml EGF for 15 min. Cell lysates were subjected to Western blot analysis using the antibodies against human AKT and Ser^{473}-phosphorylated AKT, respectively. SNCG overexpression didn't affect E_2 and EGF-induced AKT phosphorylation.

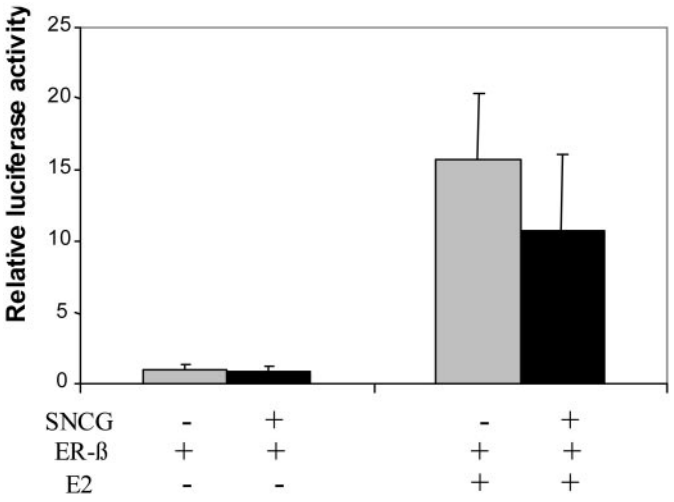

Fig. 7. Effects on transcriptional activity of ER-β. MCF-7 cells were transiently cotransfected with pSG5-hERβ and pCI-SNCG or the control vector pCI-neo. After selection with G418, the transfected cells were transfected with pERE4-Luc, as well as control reporter pRL-SV40-Luc, cultured in the ligand-free medium for 4 days, treated with or without 1 nM estradiol (E_2) for 24 h before harvesting. The promoter activities were determined by measuring the dual luciferase activity. All values were presented as the fold induction over the control luciferase activity in the nontreated SNCG-negative cells, which was taken as 1. The numbers represent means ± SD of three cultures.

Stimulation of Estrogen-Dependent Mammary Tumorigenesis by SNCG. An orthotopic nude mouse model was used to study the effects of SNCG on tumor growth. Two independent experiments under different conditions were done to determine the effects of SNCG on mammary tumorigenesis. We first analyzed the tumorigenesis in response to E_2 in nonovariectomized mice. It was previously demonstrated that the circulating E_2 level in the nonovariectomized mice is about 26 pg/ml (30), which compares to the low levels found in postmenopausal women (31). To override the endogenous levels of estrogen, all of the mice were supplemented with E_2 (0.72 mg/pellet) 1 day before injection of 5×10^6 cells. After a lag phase of 8–10 days, 29 of 32 (91%) injections in the mice given implants of SNCG-positive MCF-SNCG2 and MCF-SNCG6 cells developed tumors. In contrast, only 21 of 32 (66%) injections in the mice given implants of SNCG-negative MCF-neo1 and MCF-neo2 cells developed tumors (Table 1). The tumor growths in MCF-SNCG clones were significantly stimulated. At 35 days after tumor cell injection, the size of MCF-SNCG6 tumors, which expressed a relative high level of SNCG mRNA, was 4.8-fold of that in parental MCF-neo2 tumors and 3.7-fold of that in MCF-neo1 tumors. In addition, the tumor incidence was also increased. With 16 injections, whereas 15 implants in MCF-SNCG6 cells developed tumors, only 10 implants from MCF-neo1 and 11 implants from MCF-neo2 developed tumors, respectively. The tumor growth of MCF-SNCG2 cells was also significantly stimulated, with 3.5- and 2.7-fold increase in tumor size as compared with MCF-neo2 and MCF-neo1 tumors, respectively. Fig. 8 shows growth kinetics. After a slow growth phase of 14 days, tumor growth of MCF-SNCG2 and MCF-SNCG6 clones were significantly enhanced as compared with that of MCF-neo1 and MCF-neo2 clones. Thus, the tumorigenesis of MCF-7 cells in response to E2 was significantly stimulated by SNCG overexpression.

We then analyzed the requirement of E_2 for SNCG-mediated tumor

Table 1 *Effects of SNCG expression on tumor incidence and tumor growth of MCF-7 cells*[a,b]

		Tumor incidence	Tumor volume (mm^3)	
Experiment group	E_2	Tumor/Total(%)	Day 21	Day 35
MCF-SNCG2	+	14/16 (88)	234 ± 69	1850 ± 280
MCF-SNCG6	+	15/16 (94)	351 ± 78	2521 ± 390
MCF-neo1	+	10/16 (63)	101 ± 39	491 ± 92
MCF-neo2	+	11/16 (69)	123 ± 25	625 ± 130

[a] Cells were injected into the mammary fat pads, and tumor volumes and tumor incidence were determined as described in "Materials and Methods." Each mouse received two injections. Tumor volumes were measured at 21 and 35 days after cell injection and are expressed as means ± SEs (number of tumors assayed). All the nonovariectomized mice received an estrogen implantation 1 day before the cell injection. There were total 16 injections for 8 mice in each group, and each injection had 5×10^6 cells. Statistical comparisons for SNCG-positive clones relative to SNCG-negative clones indicated $P < 0.01$ for the mean tumor sizes and $P < 0.05$ for the tumor incidence. Statistical comparison for primary tumors was analyzed by Student's *t* test. A χ^2 test was used for statistical analysis of tumor incidence.

[b] SNCG, γ synuclein; E_2, estradiol.

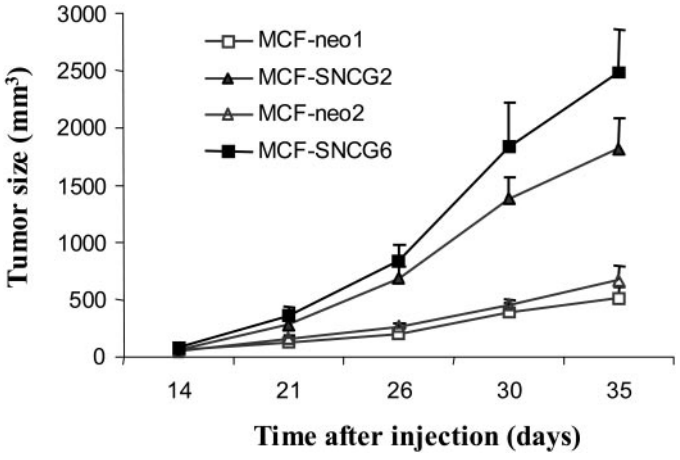

Fig. 8. Stimulation of MCF-7 tumor growth by γ synuclein (SNCG). Each of the eight estradiol-supplemented nonovariectomized mice in each group received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each clone at each time point. Each point represents the mean of tumors ± SE (*bars*).

Table 2 *Stimulation of estrogen-mediated tumorigenesis by SNCG*[a,b]

Group	E_2	Tumor incidence (%)							Tumor volume (mm^3)
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 7
MCF-neo1	-	0	0	0	0	0	0	0	
MCF-neo1	+	0	0	0	0	20	30	30	44 ± 10
MCF-SNCG6	-	0	0	0	0	0	0	0	
MCF-SNCG6	+	40	80	90	90	90	90	90	194 ± 35

[a] Ovariectomized mice were treated with or without E_2 pellet. There were total 10 injections for 5 mice in each group, and each injection had 1.5×10^6 cells. Only measurable tumors were used to calculate the mean tumor volume. Statistical comparisons for SNCG-positive clones relative to SNCG-negative clones indicated $P < 0.001$ for both tumor incidence and mean tumor sizes in the presence of E_2.
[b] SNCG, γ synuclein; E_2, estradiol.

stimulation under more stringent conditions, including use of ovariectomized mice and reduction of injected tumor cells from 5×10^6 to 1.5×10^6. As shown in Table 2, when the number of injected MCF-neo1cells was reduced, the tumor incidence was greatly reduced from 63% to 0%, both in the absence and presence of E_2 supplement for up to 4 weeks, indicating a nontumorigenic condition for SNCG-negative MCF-7 cells even with estrogen stimulation for 4 weeks. At week 7, a 30% of tumor incidence with small tumor size (44 mm^3) was observed. However, for MCF-SNCG6 cells, although the same nontumorigenic phenotype was observed under the conditions with reduced cell number and in the absence of E_2 when E_2 was supplemented, the tumor incidence reached 90% 3 weeks after cell inoculation, which is similar to 94% of tumor incidence in the experiment with higher injected cell numbers (Table 1). Furthermore, the tumor size of SNCG-positive cells is 4.4-fold of control. These data indicate that estrogen is necessary for SNCG-mediated tumor stimulation in this xenograft model.

DISCUSSION

Synucleins are small proteins expressed predominately in neurons and have been specifically implicated in neurodegenerative disorders such as AD and PD. However, *SNCG* was first identified and cloned as a breast cancer specific gene, which is highly expressed in advanced infiltrating breast carcinomas but not in normal or benign breast tissues (1). Aberrant expression of *SNCG* was also associated with ovary cancer progression (4, 19). The association between SNCG expression and the progression of steroid-dependent cancers of breast and ovary suggest a potential role of SNCG in regulation of ER-α. Previously, we demonstrated a SNCG-stimulated ER-α signaling in three different cell systems, including (*a*) overexpression of SNCG in ER-α-positive and SNCG-negative MCF-7 cells, (*b*) antisense blockage of SNCG expression in ER-α-positive and SNCG-positive T47D cells, and (*c*) cotransfection of SNCG and ER-α into SNCG-negative and ER-α-negative MDA-MB-435 cells (16). The results shown in this study demonstrated that SNCG stimulated mammary tumorigenesis in response to estrogen, which is mediated by its participation in Hsp-based chaperone complex for regulation of ER-α transcriptional activity.

To acquire the ability to bind hormone, steroid hormone receptors undergo a series of transformation steps in which they are brought into the correct conformation by molecular chaperones and cochaperones. The most extensively studied chaperones for steroid receptors are a multiprotein Hsp70- and Hsp90-based chaperone system, which includes Hsp90, Hsp70, Hop, Hsp40, p23, and others (23, 24). Hsp70 and Hsp90 associate with the unliganded steroid hormone receptors and maintain the conformational state for efficient ligand binding and receptor activation. Consistent with the previous report on chaperone-like activity of synucleins (27), here, we provided evidences suggesting that SNCG is a new member of molecular chaperone proteins that participates in Hsp-based chaperone complex for regulating ER-α activity. These evidences include that (*a*) SNCG bound to the unliganded form of ER-α, Hsp90, and Hsp70, (*b*) SNCG enhanced the high-affinity ligand-binding state of ER, and (*c*) SNCG significantly stimulated the transcriptional activity of ER-α and ligand-dependent mammary tumorigenesis. The binding of SNCG to ER-α and Hsp90 only occurs in the absence of ligand, which is same to the binding of Hsp90 and Hsp70 to the unliganded ER-α. However, the binding between SNCG and Hsp70 was observed under the conditions both with and without the ligand, suggesting that SNCG is an Hsp70-binding protein.

It has been previously demonstrated in the cell-free system that Hsp70-free reticulocyte lysate is inactive at glucocorticoid receptor heterocomplex formation with Hsp90 and that the activity is restored by readdition of purified Hsp70 (32, 33), indicating that the binding of Hsp70 to the unliganded steroid receptor is necessary for the efficient Hsp90 binding for maintaining the high transcriptional activity of steroid receptors. Hsp90 is absolutely essential for hormone binding to glucocorticoid receptor under all conditions (34). Recent studies from *in vivo* yeast system and *in vitro* mammalian cell-free system also indicate that ER requires the molecular chaperone Hsp90 for efficient hormone binding (35). There were two pools of ER, one with high hormone affinity and one with low affinity (Fig. 4). Although the nature of the low-affinity state is unclear, its existence in cells may reflect an equilibrium between the receptors with high hormone affinity and poised for activation and those with low affinity. The low-affinity hormone-binding state may reflect a kinetically trapped folding intermediate that requires the action of SNCG, Hsp90, and other proteins for the conversion into the high-affinity state. A working model for the role of SNCG on ER-α transcriptional activation is presented in Fig. 9. In this model, SNCG binds to Hsp70 and forms a complex including Hsp90, Hsp70, SNCG, ER-α, and others. This

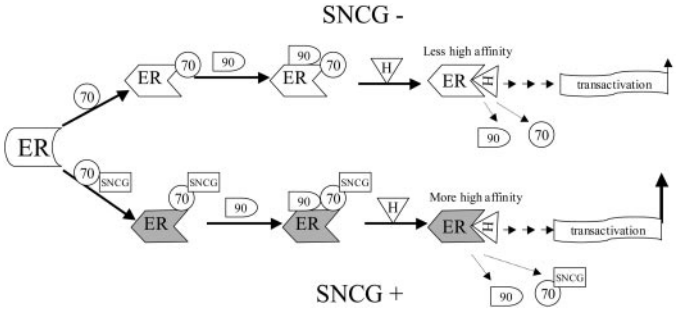

Fig. 9. A model for γ synuclein (SNCG)-regulated estrogen receptor (ER)-α activation. In the model, 90 refers to heat-shock protein (Hsp)90, 70 refers to Hsp70, H refers to hormone. According the model, SNCG and Hsp70 preassociate to form SNCG-Hsp70 chaperone complex. This complex binds to the unstable hormone-binding domain of the native receptor. The recruitment of Hsp90 to the ER-α would occur once the receptor was bound with the SNCG-Hsp70 complex. At this stage, the receptor undergoes structure changes and assumes a conformation with high affinity for estradiol. Upon the binding to estradiol, the receptor disassociates with SNCG-Hsp70 complex and Hsp90, which leads to receptor dimerization, interaction with coactivators, and transactivation.

chaperone complex pushes the equilibrium toward the high-affinity hormone-binding conformation. Although the nature of the conformational changes in ER-α is unknown, SNCG overexpression would be expected to result in a greater number of mature ER complexes with high-affinity ligand-binding capability. As a result, the physiological levels of E_2 binding will increase, which is consistent with the E_2-binding assays (Figs. 3 and 4). Thus, overexpression of SNCG, by increasing the number of mature receptor complexes with high-affinity ligand-binding capability, will manifest itself as an increase in transcriptional activation by ER at a given hormone concentration.

One of the critical questions need to be addressed is whether SNCG-stimulated ER-α transactivation is mediated by a Hsp-based chaperone complex, particularly Hsp70. Using Hsp90 inhibitor GA, our data demonstrated that treatment of cells with GA completely abolished SNCG-stimulated receptor ligand binding, indicating that the SNCG-mediated stimulation of ER-α is Hsp dependent. However, this study needs to be additionally confirmed with more specific approaches such as small interfering RNAs to knock out Hsp70 or Hsp90. We realize that Hsp70 and Hsp90 play a critical role in maintaining ER-α in a right conformation for ligand binding. Without chaperone activity of Hsp70 and Hsp90, the transcriptional activity of ER-α might not be efficiently activated. In this regard, inhibition of endogenous levels of Hsp70 or Hsp90 by small interfering RNA may greatly affect ER-α transactivation, and thus, the SNCG-mediated stimulation of ER-α transactivation may be jeopardized. SNCG may also regulate ER-α signaling by Hsp-independent pathways such as direct binding to and chaperoning ER-α. However, our *in vitro* translation study indicates that SNCG did not physically interact with ER-α directly (data not shown). Nevertheless, our model suggests that SNCG-stimulated transcriptional activity of ER-α is mediated, at least in part, by participating in Hsp-based multiprotein chaperone complex and maintaining the ER-α in a high-affinity hormone-binding conformation. Thus, SNCG and Hsps act cooperatively in ER-α signaling.

The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. It has been demonstrated in different systems that the interaction between SNCG and ER-α stimulated cell growth in response to estrogen. First, although expression of SNCG in MCF-7 cells had no effect on the cell growth in the absence of E_2, SNCG significantly stimulated the ligand-dependent cell growth, which can be blocked by antiestrogens (16). This growth stimulation was also previously demonstrated in the anchorage-independent growth assay (15). Second, when endogenous SNCG expression in T47D cells was blocked by expressing SNCG antisense mRNA, the anchorage-independent growth in response to E_2 was significantly suppressed in the cells expressing antisense SNCG (16). Third, although the alteration of SNCG expression affected the cell growth of ER-α-positive MCF-7 and T47D cells, it had no effect on the cell growth of ER-α-negative MDA-MB-435 cells (14). Finally, SNCG overexpression significantly stimulated the tumorigenesis of MCF-7 cells in response to estrogen, whereas it has no effect on tumor growth in the absence of estrogen. Consistent with the requirement of E_2 for SNCG-stimulated tumor growth, it was demonstrated that SNCG had no significant effect on tumor growth of ER-α-negative MDA-MB-435 cells (14).

We isolated a 2195-bp promoter fragment of a human *SNCG* gene and demonstrated that demethylation of exon 1 region of *SNCG* gene is an important factor responsible for the aberrant expression of SNCG in breast carcinomas (21, 22). However, the molecular targets of SNCG aberrant expression in breast cancer have not been identified. Our findings suggest that SNCG functions as a chaperone and participates in Hsp-based multiprotein chaperone system for efficient activation of ER-α. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression, at least in part, by enhancing the transcriptional activity of ER-α. The role of SNCG in breast cancer progression may also involve other non-ER-mediated functions such as stimulation of tumor motility and metastasis as we previously described in hormone-independent breast cancer cells (14).

The cellular functions of synucleins remain elusive. Although the chaperone-like activity has been suggested for synucleins based on the cell-free system (27), the molecular targets for chaperone activity remain to be identified. Recently, the protective effect of molecular chaperone Hsp70 on SNCA-induced dopaminergic neuronal loss in *Drosophila* has been reported (36), indicating that chaperone activity of Hsp70 helps to protect neurons against the neurotoxic consequences of SNCA expression. Interestingly, although filamentous SNCA is the major deposit in intracellular inclusions in neurons, SNCG and SNCB inhibit SNCA fibril formation, suggesting a protective effect of SNCG and SNCB on SNCA aggregation (37). SNCG-mediated chaperone activity on ER-α may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG may be involved in regulating Hsp70 and mediating the activation of ER-α in neuronal cells; thus, the down-regulation of SNCG expression may lower the beneficial effects of estrogen on protecting neurons against PD and AD. The potential role of SNCG as a neuroprotectant warrants additional investigation. Demonstration of direct interaction with ER-α chaperone complex and stimulation of ER-α signaling as one of the cellular functions of SNCG not only support its pathological role in the growth of steroid-responsive tumors but may also shed some light on the cellular functions of synucleins in brain cells and their complex roles in the development of neurodegenerative disorders.

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Advances in Brief

Stimulation of Estrogen Receptor Signaling by γ Synuclein[1]

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Abstract

Synucleins are emerging as central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's disease and Parkinson's disease. However, γ Synuclein (SNCG) is also highly associated with breast cancer and ovarian cancer progression. Whereas most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue, the normal cellular function of this highly conserved synuclein family remains largely unknown. A notable finding in this study is that SNCG, identified previously as a *breast cancer-specific gene 1*, strongly stimulated the ligand-dependent transcriptional activity of estrogen receptor-α (ER-α) in breast cancer cells. Augmentation of SNCG expression stimulated transcriptional activity of ER-α, whereas compromising endogenous SNCG expression suppressed ER-α signaling. The SNCG-stimulated ER-α signaling was demonstrated in three different cell systems including ER-α-positive and SNCG-negative MCF-7 cells, ER-α-positive and SNCG-positive T47D cells, and SNCG-negative and ER-α-negative MDA-MB-435 cells. The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. Whereas overexpression of SNCG stimulated the ligand-dependent cell proliferation, suppression of endogenous SNCG expression significantly inhibited cell growth in response to estrogen. The stimulatory effect of SNCG on ERα-regulated gene expression and cell growth can be effectively inhibited by antiestrogens. These data indicate that SNCG is required for efficient ER-α signaling and, thus, stimulated hormone-responsive mammary tumors.

Introduction

Synucleins are a family of small proteins consisting of 3 known members, SNCA,[3] SNCB, and SNCG. Synucleins has been specifically implicated in neurodegenerative diseases such as AD and PD. Mutations in SNCA are genetically linked to several independent familial cases of PD (1). More importantly, wild-type SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (2, 3). SNCA peptide known as nonamyloid component of plaques has been implicated in amyloidogenesis in AD (4, 5). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy body cases (6, 7). Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases, particularly in the hormone-responsive cancers of breast and ovary (8–13).

We have reported previously the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using differential cDNA sequencing approach (8, 14). Of many putative differentially expressed genes, a breast cancer-specific gene, *BCSG1,* was identified as a putative breast cancer-specific gene, which was highly expressed in a breast cancer cDNA library but scarcely in a normal breast cDNA library (8). Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neural protein synuclein, having 54% and 56% sequence identity with SNCA and SNCB, respectively. Subsequent to the isolation of *BCSG1*, *synuclein* γ (13) and *persyn* (15) were cloned independently from a brain genomic library and a brain cDNA library. In fact, BCSG1, SNCG, and persyn appear to be the same protein. Thus, the previously identified BCSG1, which is also highly expressed in brain, has been renamed as SNCG (16).

Although synucleins are abundant proteins expressed in presynaptic terminals and tightly associated with amyloid plaque in AD and Lewy body in PD, the normal cellular function of this highly conserved synuclein family remains largely unknown. Being identified as a breast cancer-specific gene, SNCG expression in breast follows a stage-specific manner: SNCG was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but expressed at an extremely high level in advanced infiltrating breast cancer (8, 11). Overexpression of SNCG in cancer cells led to significant increase in cell motility and invasiveness *in vitro*, profound augmentation of metastasis *in vivo* (9), and resistance to chemotherapeutic drug-induced apoptosis (17). Overexpression of synucleins, especially SNCG and SNCB, also correlated with ovarian cancer development (11, 13). Whereas synuclein (α, β, and γ) expression was not detectable in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express either SNCG or SNCB, and 42% (19 of 45) expressed all three of the synucleins (α, β, and γ) simultaneously (11). The involvement of SNCG in hormone-responsive cancers of breast and ovary prompted us to explore the potential role of SNCG in cellular response to estrogen. In the present study, we evaluated the biological functions of SNCG on regulation of estrogen-receptor transcriptional activity in human breast cancer cells. The results suggest that one of the critical functions of SNCG on breast cancer pathogenesis is to stimulate ER-α transcriptional activity.

Materials and Methods

Conditioned Cell Culture. All of the cell lines used in this study (MCF-7, T47-D, and MDA-MB-435) were originally obtained from the American Type Culture Collection. Proliferating subconfluent human breast cancer cells were harvested and cultured in the phenol red-free IMEM containing 5% charcoal-stripped FCS for 4 days before addition of indicated dose of E2. Cells in the absence or presence of E2 were collected 24 h after addition of E2 and were subjected to the assays for ER-α transcriptional activity.

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[3] The abbreviations used are: SNCA, α synuclein; AD, Alzheimer's disease; BCSG1, breast cancer specific gene 1; ER, estrogen receptor; Cat-D, cathepsin D; Hsp, heat shock protein; PD, Parkinson's disease; PR, progesterone receptor; SNCB, β synuclein; SNCG, γ synuclein; ERE, estrogen response element; RT-PCR, reverse transcription-PCR; E_2, 17β-estradiol; TGF, transforming growth factor; ERE4-Luc, ERE4-Luciferase; ICI, ICI 182,780.

Gene Transfection. Subconfluent proliferating cells in 12-well plate were incubated with 2 μg of expression vectors in 1 ml of serum-free IMEM containing LipofectAMINE for 5 h. Culture was washed to remove the excess vector and LipofecTAMINE, and then postincubated for 24 h in fresh culture medium to allow the expression of transfected gene.

Assays for the Transcriptional Activity of ER-α. Cells were transiently transfected with a firefly luciferase reporter construct (pERE4-Luc) containing four copies of the ERE (18). For the cotransfection experiments, the plasmid DNA ratio of pERE4Luc to expression vectors of ER-α or SNCG was 2:1. A renilla luciferase reporter, pRL-SV40-Luc, was used as an internal control for transfection efficiency. Luciferase activities in total cell lysate were measured using the Promega Dual Luciferase Assay System. Absolute ERE promoter firefly luciferase activity was normalized against renilla luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition, and at least three independent transfection assays were performed.

RT-PCR Analysis. MCF-7-derived cells were cultured in ligand-free medium for at least 5 days, and treated with 10^{-9} M E_2 for 4 h as indicated. Total RNA from cells were isolated using RNeasy Kit (Qiagen Inc.). Approximately 4 μg of total RNA was subjected to semiquantitative RT-PCR analysis following a procedure described previously for estrogen-responsive genes (19, 20). The primer sequences (5′-3′) are as follows: TGF-α, sense CGCCCTGTTCGCTCTGGGTAT, antisense AGGAGGTCCGCATGCTCACAG (240-bp product); cathepsin-D, sense CCAGCCCCCAATCCCAACCCCACCTCCAG, antisense ACTGAAGCTGGGAGGCAAAGGCTACAAGC (842-bp product); and PS2, sense CATGGAGAACAAGGTGATCTG, antisense CAGAAGCGTGTCTGAGGTGTC (336-bp product).

Stable Expression of SNCG Antisense mRNA in T47D Cells. A 285-bp DNA fragment corresponding to the exon 1 region (−169 to +116) of SNCG gene was amplified from the plasmid pBS-SNCG759 and was cloned into the *EcoR*I site of the expression vector pcDNA3.1. The antisense or sense orientation of the exon 1 in the pcDNA3.1 vector was determined by restriction enzyme digestion and was verified by DNA sequencing. Vectors expressing SNCG antisense mRNA (pcDNA-SNCG-As) or SNCG sense mRNA (pcDNA-SNCG-S) were transfected separately into T47D cells by Effectin reagent. Isolated clones were picked up after G418 selection. The expression of SNCG antisense and sense mRNAs (285 bp) in the individual clones was confirmed by RT-PCR reaction. For antisense mRNA, the primer sets are: T7 as the forward primer, 5′ TAATACGACTCACTATAGGG 3′ and SNCG-Wf as the reverse primer, ACGCAGGGCTGGCTGGGCTCCA. The primer sets for detection of sense mRNA are: T7 as the forward primer, 5′ TAATACGACTCACTATAGGG 3′ and SNCG-Wr as the reverse primer, 5′ CCTGCTTGGTCTTTTCCACC 3′.

Cell Proliferation Assay. For [^{3}H]thymidine incorporation, cells were cultured and synchronized in the conditioned medium for 4 days as described in "Conditioned Cell Culture." Cells were treated with or without 1 nM of E_2 for 24 h. [^{3}H]Thymidine was added 12 h before harvesting. [^{3}H]Thymidine incorporation was determined by precipitation with 10% trichloroacetic acid followed by liquid scintillation counting. Triplicate wells were assayed for each cellular proliferation condition, and at least three independent assays were performed. Cell growth was also measured using a cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, exponentially growing cells were seeded in quadruplicate at 1500 cells per well (96-well plate) in the conditioned medium. Cells were treated with indicated chemicals for 6 days before harvesting.

Soft Agar Colony Formation Assay. The anchorage-independent growth was carried out in 12-well plates as we described previously (10). The bottom layer consists of 0.5 ml of 5% charcoal-striped calf serum/IMEM containing 0.6% agar. The top layer consists of 0.25 ml of 5% charcoal-striped calf serum/IMEM containing 0.4% agar and ~2000 cells. In the E_2-treated groups, the top layer also contains 1 nM of E_2. Cells were cultured under high humidity condition. Cells were fed with 0.1 ml of culture medium with or without E_2 every 4 days. After 2 weeks, the number of colonies in each well was counted under a Nikon microscope at ×100 amplification. Triplicate wells were assayed for each condition.

Results

Overexpression of SNCG Stimulated Transcriptional Activity of ER-α. Estrogen response is mediated by two closely related members of the nuclear receptor family of transcription factors, ER-α and ER-β (21, 22). Because ER-α is the major ER in mammary epithelia, we measured the effect of SNCG on modulating the transcriptional activity of ER-α in human breast cancer cells. We first selected ERα-positive and SNCG-negative MCF-7 cells as recipients for SNCG transfection (Fig. 1, *A* and *B*). MCF-7 cells were transiently transfected with either the pCI-SNCG expression plasmid or control pCI-neo plasmid. Transfection of the *SNCG* gene into the SNCG-negative MCF-7 cells did not affect ER-α expression under the conditions both with and without E_2 (Fig. 1*A*). In the absence of E_2, the basal levels of ER-α on control and SNCG-transfected cells are the same. Although treatment of the control cells with E_2 resulted in a significant decrease in ER-α level, overexpression of SNCG did not affect E_2-mediated degradation of ER-α. Transfection of SNCG significantly stimulated E_2-mediated activation of ER-α (Fig. 1*B*). Treatment of wild-type and SNCG-transfected MCF-7 cells with E_2 resulted in a significantly differential increase in estrogen-responsive reporter ERE4-Luc activity relative to basal levels in untreated cells. Overexpression of the *SNCG* gene in MCF-7 cells increased E_2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ER-α was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ER-α in the absence of E_2.

Consistent with the increased transcriptional activity of ER-α,

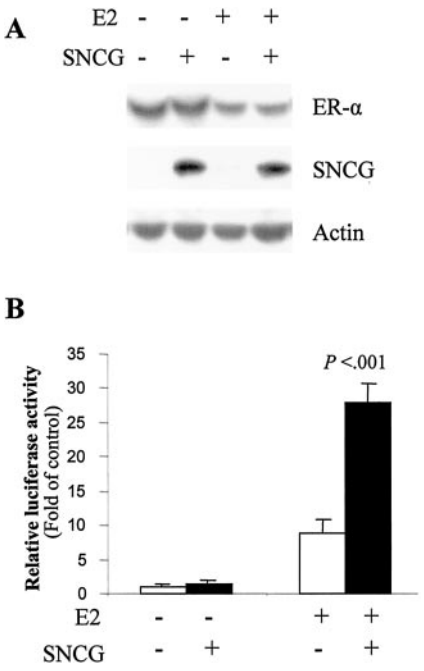

Fig. 1. SNCG stimulated ER-α transcriptional activity in MCF-7 human breast cancer cells. Cells were first transiently transfected with pCI-SNCG or the control vector pCI-neo. The transfected cells were selected with G418 and then transfected with pERE4-Luc, as well as control reporter pRL-SV40-Luc. After transfection, cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods," treated with or without 1 nM E2 for 24 h before the promoter activities were determined by measuring the dual luciferase activity (*A*). Western analysis of ER-α and SNCG in MCF-7 cells transfected with pCI-SNCG or the control vector pCI-neo. Expression of SNCG did not affect the ER-α expression in the conditions both with and without 24-h E_2 treatment. SNCG stimulated ER-α signaling in MCF-7 cells (*B*). The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the nontreated SNCG-negative cells, which was taken as 1. The numbers represent means of three cultures; *bars,* ±SD.

SNCG also stimulated E_2-regulated genes in MCF-7 cells (Fig. 2). Whereas SNCG had no effect on the transcription of Cat-D, PS2, and TGF-α in the absence of E_2, transcription of Cat-D, PS2, and TGF-α were increased 3.9-fold, 3.2-fold, and 4.2-fold in SNCG transfected cells *versus* control cells in the presence of E_2, respectively (Fig. 2*A*). To evaluate the effect of antiestrogen on SNCG-stimulated ER-α-regulated genes, we treated the cells with an antiestrogen ICI. As demonstrated in Fig. 2*B,* the basal levels of PR were very weak in both SNCG-transfected and control cells, but were increased significantly by E_2 treatment. Treatment of the cells with E_2 stimulated a 3.5-fold PR protein expression in SNCG-transfected cells compared with control cells. Although ICI slightly stimulated basal levels of PR, treatment of the SNCG-transfected MCF-7 cells with ICI significantly blocked E_2-stimulated PR expression, indicating that SNCG-stimulated gene expression in E_2-treated cells is mediated by ER-α.

We also investigated the effect of SNCG on the transcriptional activity of ER-α in ERα-negative and SNCG-negative MDA-MB-435 breast cancer cells (Fig. 3). Treatment of ER-α-transfected MDA-MB-435 cells with E_2 activated reporter activity, indicating the functional transcriptional activity of the transfected ER-α gene. A significant stimulation of ER-α signaling by SNCG was observed in MDA-MB-435 cells when the cells were cotransfected with ER-α and SNCG constructs. SNCG increased ligand-dependent transcriptional activity 3.7-fold over the control cells.

Reduced Levels of SNCG Compromised Transcriptional Activity of ER-α. The effect of SNCG expression on ER-α transactivation was additionally demonstrated by inhibiting endogenous SNCG expression with SNCG antisense mRNA in T47D cells that express high levels of SNCG (8). Stable transfection of the SNCG antisense construct into T47D cells significantly reduced SNCG expression to 25% of that in control T47D cells (Fig. 4*A*). Whereas E_2 significantly stimulated the reporter activity in the control T47D cells, inhibition of SNCG expression reduced E_2-responsive luciferase activity in two stable antisense-transfected T47D cell lines, AS-1 and AS-3 cells, to 21% and 13% of that in control T47D cells, respectively (Fig. 4*B*). Treatment of T47D cells with E_2 resulted in a 25-fold increase over the nontreated cells. However, only 3.3- and 5.2-fold increase was observed in the AS-3 and AS-1 cells, respectively. Taken together, the increased estrogen-responsive reporter activity in SNCG-transfected MCF-7 and MDA-MB-435 cells, as well as the increased estrogen-regulated gene transcription and the compromised transcriptional activity of ER-α in SNCG antisense-transfected T47D cells indicated that SNCG stimulated ligand-dependent transcriptional activity of ER-α.

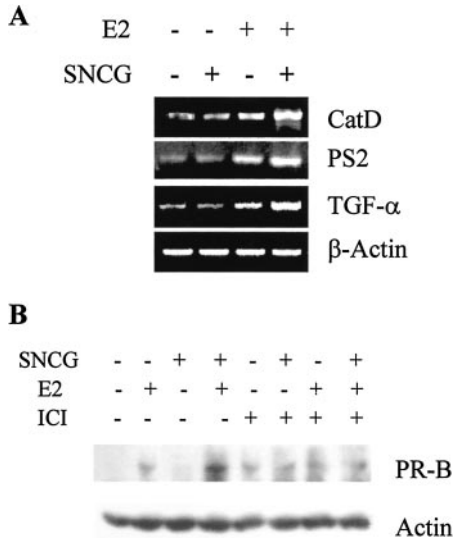

Fig. 2. SNCG stimulated estrogen-regulated gene transcription in MCF-7 cells. Cells were transiently transfected with pCI-SNCG or the control vector pCI-neo. After G418 selection, cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods." *A,* RT-PCR. Cells were treated with or without 1 nM of E2 for 8 h before the isolation of total RNA. Expressions of mRNA of Cat-D, PS2, and TGF-α were studied in SNCG transiently transfected cells *versus* control cells by RT-PCR analyses. *A,* an 842-bp product of Cat-D, a 336-bp product of PS2, and a 240-bp product of TGF-α, were amplified by RT-PCR and normalized with actin. *B,* inhibition of SNCG-stimulated PR protein expression by antiestrogen ICI. Cells were treated with or without 1 nM of E_2 and 1 μM of ICI for 32 h. Total proteins were isolated, normalized, and subjected to Western analysis using anti-PR antibody.

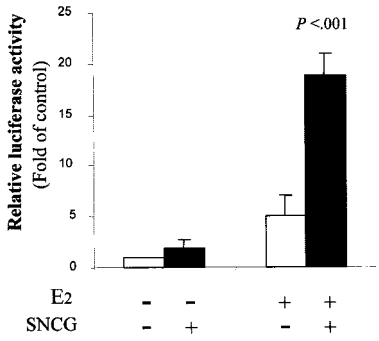

Fig. 3. SNCG stimulated ER-α transcriptional activity in MDA-MB-435 human breast cancer cells. SNCG-negative and ER-α-negative MDA-MB-435 cells were cotransfected with SNCG and ER-α constructs. Cells were cultured in the ligand-free medium for 4 days as described in the "Conditioned Cell Culture" of "Materials and Methods," treated with or without 1 nM E2 for 24 h before the promoter activities were determined by measuring the luciferase activity. The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the nontreated SNCG-negative cells, which was taken as 1. The SNCG-induced slight increase in the ligand-independent reporter activity over control was not statistically significant ($P > 0.05$); *bars,* ±SD.

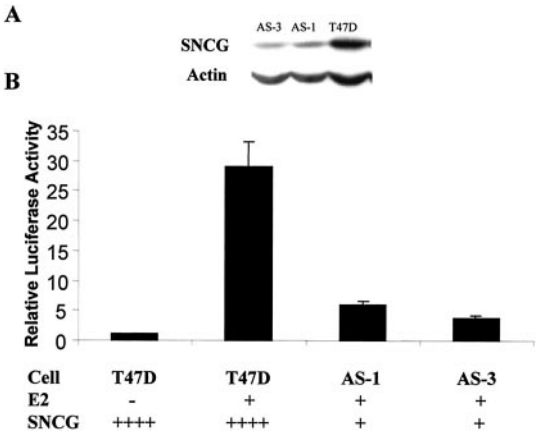

Fig. 4. Inhibition of SNCG expression reduced the transcriptional activity of ER-α. *A,* Western analysis of SNCG expression in control T47D and SNCG antisense transfected AS-1 and AS-3 cells. *B,* ERE-Luc reporter activity in control and antisense transfected T47D cells. Cells were cultured in the ligand-free conditioned medium for 4 days, treated with or without 10^{-11} M of E_2 for 24 h before harvesting. All values were normalized to the reporter activity of the nontreated T47D cells, which was set to 1. The numbers represent means of three cultures; *bars,* ±SD.

Stimulation of Cell Proliferation by SNCG. To determine the biological relevance of SNCG-stimulated ligand-dependent ER-α signaling, we analyzed the effect of SNCG overexpression on the growth of breast cancer cells. To determine whether SNCG overexpression affects ligand-dependent or ligand-independent cell growth, the cellular proliferation of the previously established two stable SNCG-transfected MCF-7 cell clones, MCF-SNCG2 and MCF-SNCG6, were compared with that of SNCG-negative cells, MCF-neo1 and MCF-neo2 (10). Data in Fig. 5*A* shows that whereas SNCG had no signif-

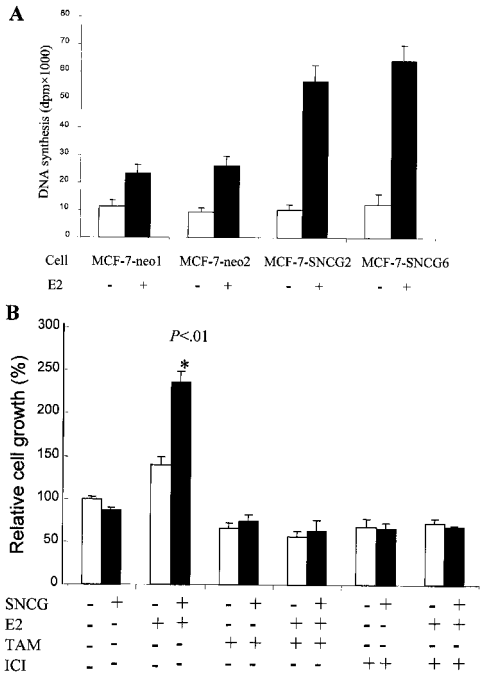

Fig. 5. SNCG stimulated ligand-dependent cell proliferation. For all experiments, cells were cultured and synchronized in the ligand-free conditioned cell culture medium for 4 days before the hormone treatments. *A,* stimulation of cell proliferation by SNCG overexpression. Cells were treated with or without 1 nM E_2 for 24 h. Cell proliferation was measured by [^{3}H]thymidine incorporation. Data are means of three cultures. *B,* effect of antiestrogens on SNCG-stimulated cell growth. Cells were treated with or without 1 nM of E_2, 1 μM of tamoxifen, or 1 μM of ICI for 6 days before harvesting. Media were changed every 2 days with fresh estrogen and antiestrogens. Cell growth was measured using a cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt). Data are the mean of quadruplicate cultures. □ represents MCF-neo1 cells; ■ represents MCF-SNCG6 cells; *bars,* ±SD.

icant effect on the proliferation of SNCG-transfected cells compared with MCF-neo cells in the absence of E_2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E_2 stimulated an average cell proliferation 2.4-fold over controls. However, E_2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation *versus* controls, suggesting that SNCG expression renders the cells more responsive to E_2-stimulated cell proliferation. To address whether the stimulatory effect of SNCG on cell growth is mediated by ER-α, we investigated the effect of the antiestrogen tamoxifen and ICI. As shown in Fig. 5*B,* E_2-stimulated growth in both MCF-neo1 and SNCG-MCF6 cells was significantly blocked by tamoxifen and ICI. These data indicate that SNCG-stimulated cell growth is mediated by ER-α.

The effect of SNCG expression on cell growth was also demonstrated in the SNCG antisense construct-transfected T47D cells. SNCG antisense mRNA expression reduced SNCG protein expression to the level of 25% of that in control T47D cells (Fig. 4*A*). Soft agar colony assays demonstrated that the anchorage-independent growth of T47D cells expressing SNCG antisense mRNA was suppressed significantly. When cells were cultured in soft agar without E_2, there were very few colonies formed in both the T47D group and T47D-SNCG antisense group. Treatment of T47D cells with E_2 resulted in a 19-fold increase of colonies over the nontreated cells. However, treatment of T47D cells expressing SNCG antisense mRNA with E_2 resulted in only 3-fold increase over the nontreated cells (Fig. 6). These data demonstrated that inhibition of endogenous SNCG expression dramatically diminished the cell growth in response to estrogen. Consistent with its stimulatory effect on ligand-dependent cell proliferation, overexpression of SNCG did not affect the proliferation of ER-α-negative MDA-MB-435 cells (9).

Discussion

Synucleins are small proteins expressed predominately in neurons, and have been specifically implicated in the neurodegenerative disorders such as AD and PD. Most studies of this group of proteins have been directed to the elucidation of their role in the formation of depositions in brain tissue. However, studies also indicated the potential role of synucleins, particularly SNCG, in the pathogenesis of steroid-responsive tumors of breast and ovary. SNCG was first identified and cloned as a breast cancer-specific gene, which is highly expressed in advanced infiltrating breast carcinomas but not in normal or benign breast tissues (8). Aberrant expression of SNCG was also associated with ovary cancer progression (11). What role SNCG has in breast and ovary, and how it is implicated in breast and ovary cancer remains a mystery. The association between SNCG expression and the progression of steroid-dependent cancers of breast and ovary led us to investigate the role of SNCG in the regulation of ER-α. Here we reported that SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α. Whereas SNCG overexpression stimulated transcriptional activity of ER-α, compromising SNCG expression suppressed ER-α signaling. The SNCG-stimulated ER-α signaling was demonstrated in three different cell systems including: (*a*) overexpression of SNCG in ER-α-positive and SNCG-negative MCF-7 cells; (*b*) antisense blocking SNCG expression in ER-α-positive and SNCG-positive T47D cells; and (*c*) cotransfection of SNCG and ER-α into SNCG-negative and ER-α-negative MDA-MB-435 cells. The results shown in this report demonstrated that human ER-α requires SNCG for efficient transcriptional activity.

The SNCG-mediated stimulation of ER-α transcriptional activity is consistent with its stimulation of the ligand-dependent cell growth. It has been demonstrated in different systems that the interaction between SNCG and ER-α stimulated cell growth. First, whereas expression of SNCG in MCF-7 cells had no effect on the cell growth in the absence of E_2, SNCG significantly stimulated the ligand-dependent cell growth, which can be blocked by antiestrogens. This growth stimulation was also demonstrated previously in the anchorage-independent growth assay (10). Second, when endogenous SNCG expression in T47D cells was blocked by expressing SNCG antisense mRNA, the anchorage-independent growth in response to E_2 was significantly suppressed in the cells expressing antisense SNCG. Third, although the alternation of SNCG expression affected the cell growth of ER-α-positive MCF-7 and T47D cells, it had no effect on the cell growth of ER-α-negative MDA-MB-435 cells (9). Consistent with the requirement of E_2 for SNCG-stimulated cell growth, we also demonstrated previously that SNCG has no significant effect on tumor growth of ER-α-negative MDA-MB-435 cells (9).

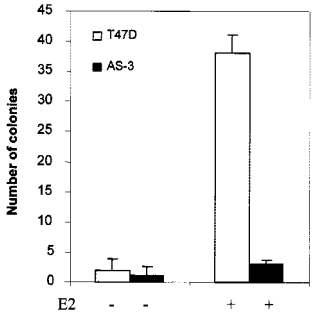

Fig. 6. Effect of inhibiting endogenous SNCG expression on soft agar colonies formation capability of T47D cells. T47D and SNCG antisense stably transfected AS-3 clone were cultured into the top layer soft agar and treated with or without 1 nM of E_2 as described in "Materials and Methods." The number of colonies was counted after 2 weeks of plating using a Nikon microscope at ×100 amplification. Triplicate wells were assayed for each condition; *bars,* ±SD.

To acquire the ability to bind hormone, steroid hormone receptors undergo a series of transformation steps in which they are brought into the correct conformation by molecular chaperones and cochaperones. The most extensively studied chaperones for steroid receptors are a multiprotein Hsp70- and Hsp90-based chaperone system, which includes Hsp90, Hsp70, Hop, Hsp40, and p23 (21–23). Hsp70 and Hsp90 associate with the unliganded steroid hormone receptors, and maintain the conformational state for efficient ligand binding and receptor activation (21, 23). Interestingly, the chaperone-like activity has been suggested for synucleins based on the cell-free system (24). However, the molecular targets for synuclein-mediated chaperone activity remain to be identified. It is likely that SNCG is a new member of molecular chaperone proteins that participate in Hsp-based chaperone complex for regulating ER-α activity. Studies are under way to investigate the mechanism by which SNCG regulates ER-α signaling.

Like SNCA of which the mutations have been detected in several cases of familial PD (1), mutations of SNCG could be linked to the development of breast carcinomas. However, after analysis of a number of breast tumors and breast cancer cell lines, it was found that the malignant phenotype correlated with the high level expression of wild-type SNCG protein (8, 11, 15). Moreover, in addition to the absence of mutation, SNCG gene amplification was also not detected in breast tumors (15). To elucidate the molecular mechanisms underlying the abnormal transcription of SNCG in breast cancer cells, we isolated a 2195-bp promoter fragment of human *SNCG* gene and demonstrated that demethylation of exon 1 region of *SNCG* gene is an important factor responsible for the aberrant expression of *SNCG* in breast carcinomas (12). However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for the action of SNCG in breast cancer pathogenesis. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression, at least in part, by enhancing the transcriptional activity of ER-α. The role of SNCG in breast cancer progression may also be involved in non-ER-mediated functions such as stimulation of tumor motility and metastasis as we described previously in hormone-independent breast cancer cells (9).

The preventive effect of estrogen on AD has become clear with epidemiological data, suggesting that estrogen may act as a neuroprotectant against the neurodegenerative diseases (25–28). The cellular functions of synucleins remain elusive. The demonstration of ER-α as the critical target for SNCG may indicate a new direction of normal cellular function of synucleins. In this regard, SNCG-mediated stimulation of ER-α signaling not only supports its pathological role in the growth of steroid-responsive tumors, but may also shed some light on the cellular functions of synucleins in brain cells and their complex roles in the development of neurodegenerative disorders.

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